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Glucose-induced hyperproliferation of cultured rat aortic smooth muscle cells through polyol pathway hyperactivity

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Abstract

Aims/hypothesis. The protein kinase C (PKC), platelet-derived growth factor (PDGF) and polyol pathway play important parts in the hyperproliferation of smooth muscle cells, a characteristic feature of diabetic macroangiopathy. The precise mechanism, however, remains unclear. This study investigated the relation between polyol pathway, protein kinase C and platelet-derived growth factor in the development of diabetic macroangiopathy.
Methods. Smooth muscle cells were cultured with 5.5 or 20 mmol/l glucose with or without an aldose reductase inhibitor, epalrestat, or a PKC-β specific inhibitor, LY333 531. Protein kinase C activities, the expression of PKC-βII isoform and PDGF-β receptor protein, free cytosolic NAD^+:NADH ratio, the contents of reduced glutathione, and proliferation activities were measured.
Results. Smooth muscle cells cultured with 20 mmol/l glucose showed statistically significant increases in protein kinase C activities, the expression of PKC-βII isoform and PDGF-β receptor protein, and proliferation activities, compared with smooth muscle cells cultured with 5.5 mmol/l glucose. Although epalrestat and LY333 531 inhibited protein kinase C activation induced by glucose to the same degree, the effects of epalrestat on proliferation activities and expression of the PDGF-β receptor were more prominent than those of LY333 531. Epalrestat improved the glucose-induced decrease in free cytosolic NAD^+:NADH ratio and reduced glutathione content, but LY333 531 did not. The increased expression of membranous PKC-βII isoform was normalized by epalrestat.
Conclusion/interpretation. These observations suggest that polyol pathway hyperactivity contributes to the development of diabetic macroangiopathy through protein kinase C, PDGF-β receptor, and oxidative stress, and that an aldose reductase inhibitor has a therapeutic value for this complication. [Diabetologia (2001) 44: 480–487]

Keywords Aldose reductase inhibitor, protein kinase C, platelet-derived growth factor, diabetic macroangiopathy, oxidative stress.

Diabetes mellitus is one of the major risk factors for atherosclerosis and is associated with an increased incidence of coronary heart diseases and cerebrovascular diseases [1, 2]. The high prevalence of these macrovascular diseases in diabetic patients can be explained by hyperglycaemia in itself [3] as well as by the increased frequency of conventional risk factors such as hypertension, hyperlipidemia, obesity, and smoking.

The proliferation of vascular smooth muscle cells is one of the characteristic features of atherosclerosis [4]. According to previous reports [4, 5], platelet-derived growth factor (PDGF) plays an important part in the accelerated proliferation of smooth muscle

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Abbreviations: ARI, Aldose reductase inhibitor; PDGF, platelet derived growth factor; PKC, protein kinase C; GSH, reduced glutathione.

cells, and it has recently been reported that smooth muscle cells from diabetic animals over-react upon PDGF stimulation, which is mediated through the overexpression of the PDGF-β receptor [5].

Hyperglycaemia leads to various metabolic abnormalities such as increased polyol pathway activity, altered protein kinase C (PKC) activities, increased oxidative stress, and enhanced non-enzymatic glycation [6]. In our previous study [7] an aldose reductase inhibitor (ARI) prevented intimal thickening in coronary arteries of galactose-fed beagle dogs, suggesting that polyol pathway hyperactivity plays an important part in the development of diabetic macroangiopathy. We also reported that epalrestat prevented the glucose-induced increase in the proliferation activities and PDGF-β receptor expression in cultured smooth muscle cells [8]. On the other hand, it has been reported that increased PKC activities contribute to the development of diabetic macroangiopathy [9] as well as nephropathy [10], retinopathy [11], and neuropathy [12], and that a PKC-β specific inhibitor can prevent these complications. In addition, the hyperglycaemia-induced increase in oxidative stress [13–16] is considered to be an important factor in the development of diabetic vascular complications [17]. Reactive oxygen species stimulate smooth muscle cell growth [18], and antioxidants, probucol, and α-tocopherol reduce the high glucose-induced proliferation of smooth muscle cells [19]. Furthermore, although previous reports [20, 21] have suggested that increases in polyol pathway activity, PKC activities, and oxidative stress are closely related to each other, the precise interrelation between these abnormalities remains controversial.

Therefore, this study investigated the relation between the polyol pathway, PKC, oxidative stress, and PDGF in the pathogenesis of diabetic macroangiopathy by measuring PKC activities, the expression of PKC-βII isoform and PDGF-β receptor protein, free cytosolic NAD^+:NADH ratio, the contents of reduced glutathione (GSH), and proliferation activities in rat aortic smooth muscle cells cultured with 5.5 or 20 mmol/l glucose in the presence or absence of a PKC-β specific inhibitor, LY333531 (11), or an aldose reductase inhibitor, epalrestat [22].

Methods and research design

Materials. Reagents were obtained from the following sources: rat aortic smooth muscle cells, A10 cells (ATCC CRL1496), from American Type Culture Collection (Rockville, Md., USA); Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin, and fetal bovine serum (FBS) from Gibco (Grand Island, N.Y., USA); sodium dodecyl sulfate (SDS) and trichloroacetic acid (TCA) from Sigma (St. Louis, Mo., USA); Whatman GF/C filter and 3 · 3 cm phosphocellulose papers (P-81) from Whatman International (Maidstone, UK); polyclonal anti-PDGF-β receptor antibody from Upstate Biotechnology (Lake Placid, N.Y., USA); antibodies for PKC-α and PKC-βII isoform from Santa Cruz Biotechnology (Santa Cruz, Calif., USA), [γ-^{32}P]-ATP from New England Nuclear (Boston, Mass., USA); epalrestat (E-3-carboxymethyl-5-(2E-methyl-3-phenylpropemylidene) rhodanine) and LY333531 were kindly provided by Ono Pharmaceutical Co. (Osaka, Japan) and Eli Lilly (Indianapolis, Ind., USA), respectively.

Cell culture. Smooth muscle cells were grown in DMEM containing 5.5 mmol/l glucose, penicillin (100 U/ml)-streptomycin (100 mg/ml), and 10% FBS, pH 7.40, at 37°C in a humidified 5% CO_2/95% air atmosphere. Third- or fourth-passage cells from the purchase allowed to grow for three weeks in DMEM containing 5.5 or 20 mmol/l glucose with or without LY333531 (2–200 nmol/l) or epalrestat (0.1–10 μmol/l) were used in the following experiments. Subculture after tripsinization was done on a weekly basis since the cells became confluent in a week. The medium was replaced with fresh medium every other day.

Assay of PKC activities. Cells were plated on 12-well plates at a density of $8 \cdot 10^3$ cells/cm^2 and grown in each experimental medium as described above. The cells were washed twice and preincubated for 1 h in serum-free DMEM. Protein kinase C activities in smooth muscle cells were measured as described previously [23] with minor modifications. Briefly, cells were rinsed twice with 2 ml of DMEM containing 20 mmol/l HEPES (pH 7.4) and then with 2 ml of salt solution (137 mmol/l NaCl, 5.4 mmol/l KCl, 0.3 mmol/l sodium phosphate, 0.4 mmol/l potassium phosphate, 5.5 mmol/l glucose, 10 mmol/l $MgCl_2$, 25 mmol/l ß-glycerophosphate, 5 mmol/l EGTA, 2.5 mmol/l $CaCl_2$, and 20 mmol/l HEPES, pH 7.4). The cells were preincubated with the salt solution for 10 min at 37°C and incubated for another 15 min in the presence or absence of 100 μmol/l PKC-specific peptide substrate, RKRTLRRL [24] with 50 μg/ml digitonin and 100 μmol/l [γ-^{32}P]-ATP (< 1500 cpm/pmol). The reaction was terminated with 5% TCA (final concentration). Aliquots of the reaction mixture were spotted on 3 · 3 cm phosphocellulose papers and washed with three changes of 75 mmol/l phosphoric acid and one change of 75 mmol/l sodium phosphate (pH 7.5). The radioactivity of phosphorylated substrate was measured by liquid scintillation counting. Protein contents were measured by the BCA method [25] and PKC activities were expressed in pmol/min/mg protein.

Assay of proliferation activities in smooth muscle cells. The proliferation activities of smooth muscle cells were assessed by the measurement of [^{3}H]-thymidine incorporation into DNA. Cells were plated on 6-well plates at a density of $10 \cdot 10^3$ cells/cm^2 and grown in each experimental medium as described above. After incubation for 5 days, the cells were preincubated with serum-free medium for 48 h, and 37 kBq/well of [^{3}H]-thymidine was added. At the end of incubation for 1 h, cells were washed with ice-cold phosphate buffered saline (PBS) three times, then dissolved with 0.1% SDS and extracted with 10% TCA. The TCA-insoluble fraction was collected on a Whatman GF/C filter and the radioactivity was measured with a liquid scintillation counter. The protein contents were measured by the BCA method [25] and the proliferation activities were expressed in dpm/mg protein.

Immunoblot analysis of PKC-α and -βII isoform protein. After incubation with each experimental medium, the cells grown on 150 mm-dishes were washed with ice-cold PBS three times, scraped with a scraper, and sonicated. Cells were then lysed in buffer A (20 mmol/l Tris-HCl, pH 7.5, 0.5 mmol/l EGTA, 2 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride,

1 mmol/l dithiothreitol, 0.3 mol/l sucrose, 25 μg/ml leupeptin, 100 μg/ml aprotinin) with Polytron for 20 s and then homogenized with 60 strokes of a Duounce homogenizer. The homogenates were centrifuged at 1000 · g for 10 min and then the supernatant was ultracentrifuged at 100000 · g for 60 min at 4 °C. The resulting supernatant was retained as the cytosolic fraction. The pellets were resuspended with buffer B (buffer A without sucrose) and homogenized again. The homogenates were solubilized in buffer B with 1 % Triton X-100 and the soluble fraction was retained as the membranous fraction after ultracentrifugation at 100000 · g for 60 min. Both the membranous and cytosolic fractions were applied to a DEAE column, washed twice with 2 ml buffer B and then finally eluted with 0.4 ml buffer B containing 200 mmol/l NaCl. After measurement of the protein concentrations, samples containing the same amount of protein (20–100 μg) were electrophoresed on SDS-PAGE (8 % acrylamide gel) and transferred to a nitrocellulose membrane. The membrane was blocked overnight with ovalbumin and incubated with a PKC-α or PKC-βII isoform antibody overnight at 4 °C followed by incubation with an anti-rabbit polyclonal IgG antibody, and visualized by ECL chemiluminesence detection kits (Amersham Pharmacia Biotech UK, Buckinghamshire, UK). Protein expressions were quantified by densitometry.

Immunoblot analysis of PDGF-β receptor protein. After incubation with each experimental medium, the cells grown on 100 mm-dishes were washed with ice-cold PBS three times, scraped, and sonicated. Cells were then lysed in a buffer containing 50 mmol/l Tris-HCl, pH 7.4, 1 % Triton X-100, 0.25 % sodium deoxycholate, 150 mmol/l NaCl, 1 mmol/l EGTA, 1 mmol/l PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mmol/l Na_3VO_4, and 1 mmol/l NaF at 4 °C. After determination of the protein concentrations, samples containing the same amount of protein (20 μg) were electrophoresed on SDS-PAGE (8 % acrylamide gel) and transferred to a nitrocellulose membrane. The membrane was blocked overnight with ovalbumin and incubated with a polyclonal anti-PDGF-β receptor antibody overnight at 4 °C followed by incubation with an anti-rabbit polyclonal IgG antibody. Receptor ligand complexes were visualized using ECL chemiluminesence detection kits (Amersham Pharmacia Biotech). Protein expressions were quantified by densitometry.

Assay of free cytosolic NAD^+:NADH ratio. The direct measurements of NAD and NADH are not informative in terms of control of metabolism because they do not provide information on compartmentalization of nicotinamide nucleotides between cytosol and mitochondria and they do not separate free from protein-bound forms (only free fractions determine direction and free-energy changes of dehydrogenase reactions). An alternative approach, implying assessment of free NAD^+:-NADH ratio in the cytoplasma by measuring the ratio of the concentrations of the oxidized and reduced metabolites of suitable NAD-linked dehydrogenase system has been proposed [26]. Using this approach, free cytosolic NAD^+:NADH ratio was calculated from metabolite concentrations and the equilibrium constant of lactate dehydrogenase as follows:

$$[NAD^+]:[NADH] = ([pyruvate]/[lactate]) \cdot 1/k_1$$

Where k_1 is the equilibrium constant of lactate dehydrogenase ($1.1 \cdot 10^{-4}$ mol/l) [27, 28]. Lactate and pyruvate were measured by enzymatic assay [29, 30].

Assay of GSH contents. The GSH contents in smooth muscle cells were assayed by the method described previously [31] with minor modifications. After incubation with each experimental medium, the cells grown on 100 mm-dishes were scraped and sonicated. Cells were lysed in cold 10 % TCA and centrifuged at 2700 rpm for 15 min. The supernatant (100 μl) was mixed with 0.89 ml of 1.0 mol/l Tris HCl, pH 8.2, containing 0.02 mol/l EDTA. To this mixture, 10 μl of DTNB (5,5'-dithio-bis 2-nitrobenzoic acid) solution (99 mg in 25 ml methanol) was added and the colored product was monitored with a spectrophotometer at 412 nm. The GSH concentration was calculated based on the standard curve (100–500 ng/ml). Protein contents were measured by the BCA method [25] and GSH contents were expressed in nmol/mg protein.

Statistical analysis. Results are presented as means ± SEM of at least three measurements for a representative experiment. Each experiment was replicated in triplicate. Differences among experimental groups were detected by analysis of variance, and the differences between groups were assessed by Bonferroni/Dunn test. Statistical significance was defined as a p value of less than 0.05.

Results

Effects of epalrestat (Figure 1A.) and LY333531 (Figure 1B.) on PKC activities in smooth muscle cells cultured with 5.5 or 20 mmol/l glucose. Protein kinase C activities in smooth muscle cells under the 20 mmol/l glucose condition (A: 34.9 ± 2.7, B: 35.4 ± 1.7 pmol/min/mg protein, $p < 0.05$) were increased compared with those under the 5.5 mmol/l glucose condition (A: 21.5 ± 1.3, B: 19.0 ± 1.7). The glucose-induced increase in PKC activities was ameliorated by epalrestat and LY333531 in a dose dependent fashion, and was almost normalized at the concentrations of 10 μmol/l (22.6 ± 0.6) and 200 nmol/l (21.1 ± 1.6), respectively. On the other hand, neither epalrestat nor LY333531 affected the PKC activities under the 5.5 mmol/l glucose condition. Therefore, these concentrations were used in the following experiments.

Effect of epalrestat on the expression of PKC-α and PKC-βII isoform protein in smooth muscle cells cultured with 5.5 or 20 mmol/l glucose. Protein kinase C isoform proteins in smooth muscle cells were characterized by using monospecific polyclonal antibodies against PKC-α or PKC-βII isoforms. The expression of PKC-βII isoform protein in the membrane fraction of smooth muscle cells cultured in 20 mmol/l glucose (162.1 ± 13.5 %, $p < 0.05$) was increased compared with that in 5.5 mmol/l glucose (100 %). Epalrestat reduced this increase in the expression of PKC-βII isoform protein in the membrane fraction (98.7 ± 4.1, $p < 0.05$) (Fig. 2). In the cytosolic fraction, there were no statistically significant differences between 5.5 and 20 mmol/l glucose in the expression of PKC-βII isoform protein with or without epalrestat. On the other hand, exposure of the smooth muscle cells to 20 mmol/l glucose did not affect the PKC-α expression in either the membrane (5.5 mmol/l glu-

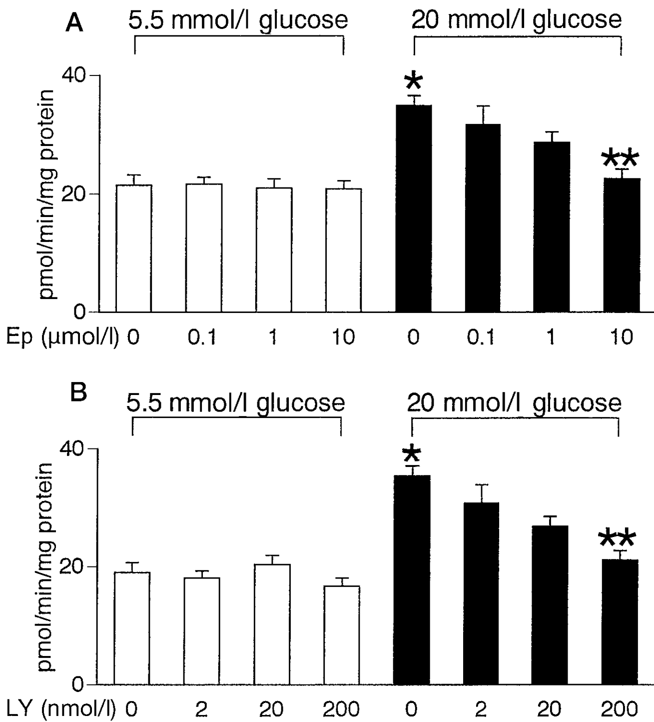

Fig. 1 A, B. Effect of epalrestat (**A**) and LY333531 (**B**) on PKC activities in smooth muscle cells cultured with 5.5 mmol/l or 20 mmol/l glucose. Values are means ± SEM of 3–6 measurements for a representative experiment. Each experiment was replicated in triplicate. LY: LY333531. Ep: epalrestat. $^{*}p < 0.05$ vs 5.5 mmol/l glucose, $^{**}p < 0.05$ vs 20 mmol/l glucose

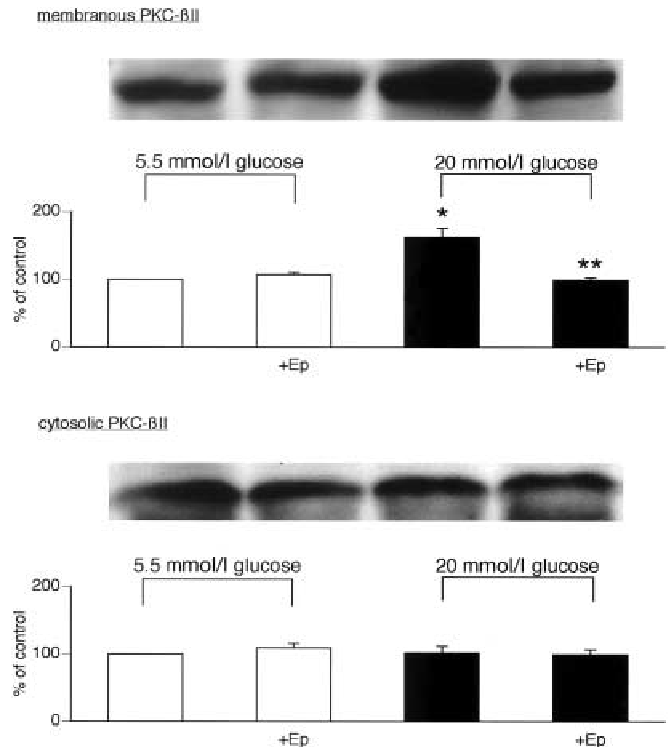

Fig. 2. Effect of epalrestat on the expression of PKC-βII protein in smooth muscle cells cultured with 5.5 mmol/l or 20 mmol/l glucose. Values are means ± SEM of 3–6 measurements for a representative experiment. Each experiment was replicated in triplicate. Ep: epalrestat (10 µmol/l). $^{*}p < 0.05$ vs 5.5 mmol/l glucose, $^{**}p < 0.05$ vs 20 mmol/l glucose

cose: 100 %, 20 mmol/l glucose: 107.8 ± 18.4) or cytosolic fraction (5.5 mmol/l glucose: 100, 20 mmol/l glucose: 108.9 ± 6.0). Epalrestat caused no changes in the PKC-α expression under 5.5 or 20 mmol/l glucose (data not shown).

Effects of epalrestat and LY333531 on proliferation activities in smooth muscle cells cultured with 5.5 or 20 mmol/l glucose. The proliferation activities in smooth muscle cells under the 20 mmol/l glucose condition ($2.31 \pm 0.08 \cdot 10^5$ dpm/mg protein, $p < 0.05$) were higher than those in the 5.5 mmol/l glucose condition (0.94 ± 0.01). Although epalrestat and LY333531 prevented the 20 mmol/l glucose-induced acceleration of proliferation activities in smooth muscle cells, this inhibitory effect of epalrestat (1.20 ± 0.11, $p < 0.05$) was more prominent than that of LY333531 (16.8 ± 0.06, $p < 0.05$) (Fig. 3). Neither epalrestat nor LY333531 altered the proliferation activities in smooth muscle cells under the 5.5 mmol/l glucose condition.

Effects of epalrestat and LY333531 on the expression of PDGF-β receptor protein in smooth muscle cells cultured with 5.5 or 20 mmol/l glucose. The expression of PDGF-β receptor protein was increased in smooth muscle cells cultured with 20 mmol/l glucose (184.7 ± 9.4 %, $p < 0.05$) compared with that cultured with 5.5 mmol/l glucose (100 %). The LY333531 ameliorated the 20 mmol/l glucose-induced acceleration of PDGF-β receptor expression (144.8 ± 6.1, $p < 0.05$). On the other hand, epalrestat normalized the increased PDGF-β receptor expression (113.7 ± 3.5), and there were statistically significant differences in the inhibitory effect between epalrestat and LY333531 (Fig. 4). Neither epalrestat nor LY333531 showed statistically significant effects on the expression of PDGF-β receptor protein in smooth muscle cells under the 5.5 mmol/l glucose condition.

Effects of epalrestat and LY333531 on free cytosolic NAD^+:NADH ratio in medium and smooth muscle cells cultured with 5.5 or 20 mmol/l glucose. Although there were no statistically significant changes in free cytosolic NAD^+:NADH ratio of the culture medium between each condition, free cytosolic NAD^+:NADH ratio in smooth muscle cells cultured with 20 mmol/l glucose was lower than that with 5.5 mmol/l glucose ($p < 0.05$). This 20 mmol/l glucose-induced decrease in free cytosolic NAD^+:NADH ratio in smooth muscle cells was ameliorated by epalrestat but not by LY333531 ($p < 0.05$) (Table 1).

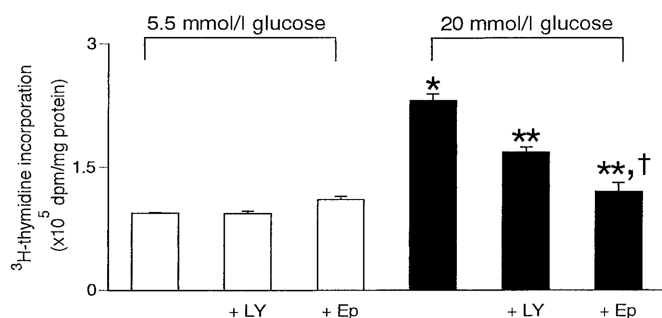

Fig. 3. Effects of epalrestat and LY333531 on proliferation activities in smooth muscle cells cultured with 5.5 or 20 mmol/l glucose. Values are means ± SEM of 3–6 measurements for a representative experiment. Each experiment was replicated in triplicate. LY: LY333531 (200 nmol/l), Ep: epalrestat (10 μmol/l). *$p < 0.05$ vs 5.5 mmol/l glucose, **$p < 0.05$ vs 20 mmol/l glucose, †$p < 0.05$ vs 20 mmol/l glucose + LY

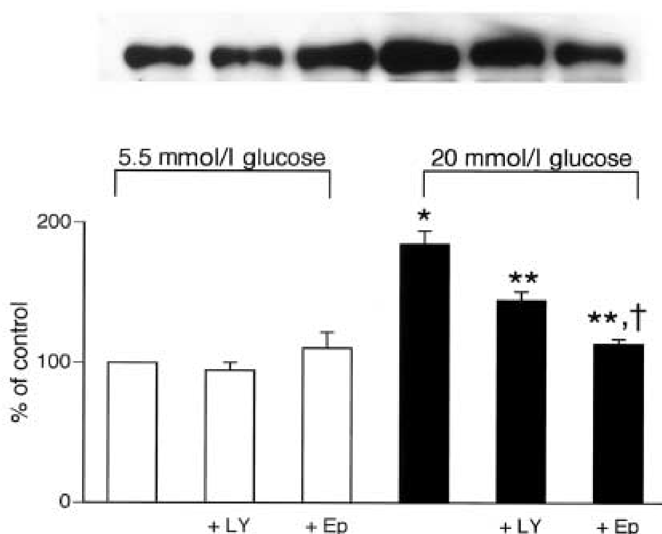

Fig. 4. Effect of epalrestat and LY333531 on the expression of PDGF-β receptor protein in smooth muscle cells cultured with 5.5 or 20 mmol/l glucose. Values are means ± SEM of 3–6 measurements for a representative experiment. Each experiment was replicated in triplicate. LY: LY333531 (200 nmol/l), Ep: epalrestat (10 μmol/l). *$p < 0.05$ vs 5.5 mmol/l glucose, **$p < 0.05$ vs 20 mmol/l glucose, †$p < 0.05$ vs 20 mmol/l glucose + LY

Effects of epalrestat and LY333531 on GSH content in smooth muscle cells cultured with 5.5 or 20 mmol/l glucose. The GSH content in smooth muscle cells cultured with 20 mmol/l glucose was decreased compared with that cultured with 5.5 mmol/l glucose ($p < 0.05$). LY333531 had no effect on the 20 mmol/l glucose-induced decrease in the GSH content, but epalrestat normalized this deficit in GSH content (Table 1). Neither LY333531 nor epalrestat altered the GSH content in smooth muscle cells cultured with 5.5 mmol/l glucose.

Table 1. Effects of epalrestat and LY333531 on free cytosolic NAD^+:NADH ratio and GSH content in medium and/or smooth muscle cells cultured with 5.5 or 20 mmol/l glucose.

	NAD^+:NADH		GSH (nmol/mg protein)
	medium	smooth muscle cells	
5.5 mmol/l glucose	4978 ± 112	4160 ± 454	5.34 ± 0.32
5.5 mmol/l glucose + LY	4978 ± 67	3900 ± 474	5.10 ± 0.55
5.5 mmol/l glucose + Ep	5139 ± 214	4169 ± 284	5.42 ± 0.74
20 mmol/l glucose	4664 ± 78	2217 ± 180[a]	2.04 ± 0.34[a]
20 mmol/l glucose + LY	4868 ± 107	2693 ± 315	2.48 ± 0.32
20 mmol/l glucose + Ep	4549 ± 186	4589 ± 724[b]	5.24 ± 0.60[b]

Values are means ± SEM of 3–6 measurements for a representative experiment. Each experiment was replicated in triplicate. LY: LY333531 (200 nmol/l). Ep: epalrestat (10 μmol/l)
[a] $p < 0.05$ vs 5.5 mmol/l glucose
[b] $p < 0.05$ vs 20 mmol/l glucose

Discussion

In this study, rat aortic smooth muscle cells cultured with 20 mmol/l glucose showed increased proliferation activities, which were accompanied by increases in PKC activities and the expression of PDGF-β receptor protein. Although an aldose reductase inhibitor, epalrestat, and a PKC-β specific inhibitor, LY333531, inhibited the glucose-induced PKC activation to the same degree, the inhibitory effect of epalrestat on the increases in proliferation activities and the expression of PDGF-β receptor was more prominent than that of LY333531. In addition, epalrestat normalized the altered redox state and accelerated oxidative stress.

In this study, the dose dependent effect of epalrestat on the polyol pathway intermediate concentrations was not studied. In our previous studies [8, 32], 100 μmol/l epalrestat completely prevented the glucose-induced sorbitol accumulation and myo-inositol depletion in cultured smooth muscle cells, and 10 μmol/l epalrestat improved these deficits. In addition, epalrestat decreased the glucose-induced hyperproliferation and overexpression of PDGF-β receptor protein in a dose dependent fashion, and normalized these abnormalities at the concentration of 10 μmol/l. Furthermore, 10 μmol/l epalrestat normalized the glucose-induced PKC activation (Fig. 1). Therefore, in this study we used 10 μmol/l epalrestat.

Increases in PKC activities have been reported in tissues of diabetic rats such as kidney, retina, aorta, and heart, or in cells cultured with high ambient glucose such as mesangial cells, smooth muscle cells, and endothelial cells [11, 33–38]. These observations suggest that hyperglycemia-induced PKC activation plays an important part in the development of diabetic complications including micro-angiopathy and macro-angiopathy. Further, among the various isoforms of PKC, the importance of preferential activation of PKC-β isoform has been confirmed in vivo by

the fact that treatment of diabetic rats or PKC-β isoform-overexpressing transgenic mice with LY333531 improved the functional and histological abnormalities in kidney and heart [10, 11, 39]. Neither the in vivo effect of LY333531 on atherosclerosis in diabetes nor the in vitro effect on the hyperproliferation of vascular smooth muscle cells cultured with high glucose has been well established. Our finding that the activities of PKC, especially PKC-βII isoform, are increased in smooth muscle cells cultured with high glucose are consistent with those of previous studies [35–37]. This increase was accompanied by hyperproliferation activities and the increased expression of PDGF-β receptor protein. Furthermore, LY333531 prevented all of these abnormalities. In addition, a recent study reported that the antisence oligonucleotide to PKC-β isoform inhibited the high glucose-induced hyperproliferation activities and overexpression of PDGF-β receptor [20]. Based on these findings, the hyperproliferation of smooth muscle cells under a high glucose condition is mediated through the PKC-β-PDGF-β receptor cascade.

The polyol pathway consists of two enzymatic reactions. The first step, glucose to sorbitol, is catalyzed by aldose reductase, and the second step, sorbitol to fructose, by sorbitol dehydrogenase. These reactions include the co-enzyme system and polyol pathway hyperactivity results in an increase in the ratio of $NADP^+$ to NADPH and a decrease in that of NAD^+ to NADH. The latter stimulates the de novo synthesis of diacylglycerol from glycolytic intermediates, resulting in PKC activation. Therefore, polyol pathway hyperactivity could be closely related with increased PKC activities. Previous studies [40, 41] have reported, however, that an ARI inhibited neither the de novo synthesis of DAG nor PKC activities in vascular cells cultured with high glucose though the sorbitol accumulation was totally prevented, and thereby concluded that polyol pathway hyperactivity did not involve the DAG-PKC pathway. In contrast, it was recently reported that an aldose reductase inhibitor showed anti-proliferative and anti-migrative effects on smooth muscle cells cultured with high glucose through the suppression of PKC-β activities [20, 42]. Our results are, in part, consistent with their observations, and suggest the importance of the polyol pathway-DAG-PKC-β-PDGF-β receptor cascade in the glucose-induced hyperproliferation of smooth muscle cells.

The most innovative observation of the present study is that the inhibitory effect of LY333531 on the hyperproliferation and overexpression of PDGF-β receptor was less prominent than that of epalrestat, which completely prevented these deficits, despite the similarity of their effects on PKC activities. This observation suggests that polyol pathway hyperactivity causes the hyperproliferation of smooth muscle cells not only through mechanisms dependent on, but also those independent of the PKC activation. LY333531 and epalrestat showed different effects on free cytosolic NAD^+ to NADH ratio and reduced glutathione (GSH) content. Neither the altered redox state (represented by free cytosolic NAD^+:-NADH ratio) nor decreased GSH content in smooth muscle cells was affected by LY333531, but epalrestat normalized these changes. As described above, although the altered redox state is located upstream of the PKC activation and related to the polyol pathway hyperactivity, the synthesis of GSH is not directly related to PKC. Therefore, the PKC-independent mechanisms could include deficits in the glutathione cycle.

Recent studies have suggested that oxidative stress increases smooth muscle cell proliferation [43] and anti-oxidants [19, 44, 45] suppress the high glucose-induced proliferation of smooth muscle cells. The increase in oxidative stress could be derived from the accelerated formation and reduced scavenging capacity of oxygen free radicals in diabetes. The glutathione redox cycle is one of the major scavenging systems of oxygen free radicals and is regulated by the intracellular contents of GSH and GSSG (oxidized glutathione), glutathione reductase activities, and NADPH concentrations [46, 47]. Aldose reductase requires NADPH as a coenzyme, and the increased polyol pathway activities lead to a depletion in NADPH, thereby competing with glutathione reductase and hampering GSH generation [48]. Therefore, it is reasonable that the polyol pathway hyperactivity would contribute to an increase in oxidative stress and the hyperproliferation of smooth muscle cells through the reduction in GSH, which was confirmed by the fact that epalrestat completely prevented the glucose-induced decrease in the GSH content in this study. In addition, it has been reported that the growth of smooth muscle cells in response to PDGF is dependent on reactive oxygen species [49], and that GSH inhibits the high glucose-induced oxidative damage and the increased PDGF secretion in cultured endothelial cells [50]. Therefore, the polyol pathway-oxidative stress-PDGF cascade independent of PKC could contribute to the glucose-induced hyperproliferation of smooth muscle cells.

Another mechanism of the glucose-induced hyperproliferation of smooth muscle cells may include the accelerated formation of advanced glycation end products (AGE). Advanced glycation end products stimulates the proliferation of smooth muscle cells [51] and the polyol pathway hyperactivity induces an acceleration of AGE formation via 3-deoxyglucosone [52]. We also reported that epalrestat prevented the increase in carlboxymethyllysin, one of the AGE, in aortas of galactosemic dogs [53]. Therefore, the effect of epalrestat observed could be at least in part mediated through the inhibition of AGE formation.

In summary, rat aortic smooth muscle cells cultured with high glucose showed statistically signifi-

cant increases in PKC activities, the expression of PKC-βII isoform and PDGF-β receptor protein, oxidative stress, and proliferation activities. Although both epalrestat and LY333531 inhibited PKC activation by high glucose to the same degree, the inhibitory effect of epalrestat on the increases in proliferation activities, the expression of PDGF-β receptor, and oxidative stress was more prominent than that of LY333531. In addition, epalrestat improved the high glucose-induced decrease in free cytosolic NAD^+:NADH ratio and GSH content, but LY333531 did not. The increased expression of PKC-βII isoform was also normalized by epalrestat. These observations suggest that the polyol pathway hyperactivity contributes to the development of diabetic macroangiopathy not only through PKC activation and overexpression of PDGF-β receptor but also through increased oxidative stress, and that an aldose reductase inhibitor has therapeutic value for this complication.

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References

1. Laakso M, Lehto S (1997) Epidemiology of macrovascular disease in diabetes. Diabetes Rev 5: 294–315
2. Goldschmid MG, Barrett-Connor E, Edelstein SL, Wingard DL, Cohn BA, Herman WH (1994) Dyslipidemia and ischemic heart disease mortality among men and women with diabetes. Circulation 89: 991–997
3. Haller H, Drab M, Luft FC (1996) The role of hyperglycemia and hyperinsulinemia in the pathogenesis of diabetic angiopathy. Clin Nephrol 46: 246–255
4. Ross R (1986) The pathogenesis of atherosclerosis. N Engl Med 314: 488–500
5. Kawano M, Koshikawa T, Kanzaki T, Morisaki N, Saito Y, Yoshida S (1993) Diabetes mellitus induces accelerated growth of aortic smooth muscle cells: association with overexpression of PDGF beta-receptors. Eur J Clin Invest 23: 84–90
6. Greene DA, Sima AA, Stevens MJ, Feldman EL, Lattimer SA. (1992) Complications: neuropathy, pathogenetic considerations. Diabetes Care 15 : 1902–1925
7. Kasuya Y, Ito M, Nakamura J et al. (1999) An aldose reductase inhibitor prevents the intimal thickening in coronary arteries of galactose-fed beagle dogs. Diabetologia 42: 1404–1409
8. Kasuya Y, Nakamura J, Hamada Y et al. (1999) An aldose reductase inhibitor prevents the glucose-induced increase in PDGF-beta receptor in cultured rat aortic smooth muscle cells. Biochem Biophys Res Commun 261: 853–858
9. Koya D, King GL (1998) Protein kinase C activation and the development of diabetic complications. Diabetes 47: 859–866
10. Koya D, Haneda M, Nakagawa H et al. (2000) Amelioration of accelerated diabetic mesangial expansion by treatment with a PKC beta inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes. FASEB J 14: 439–447
11. Ishii H, Jirousek MR, Koya D et al. (1996) Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. Science 272: 728–731
12. Nakamura J, Kato K, Hamada Y et al. (1999) A protein kinase C-beta-selective inhibitor ameliorates neural dysfunction in streptozotocin-induced diabetic rats. Diabetes 48: 2090–2095
13. Williamson JR, Chang K, Frangos M et al. (1993) Hyperglycemic pseudohypoxia and diabetic complications. Diabetes 42: 801–813
14. Sharpe PC, Yue KKM, Catherwood MA, McMaster D, Trimble ER. (1998) The effects of glucose-induced oxidative stress on growth and extracellular matrix gene expression of vascular smooth muscle cells. Diabetologia 41: 1210–1219
15. Sharpe PC, Liu WH, Yue KK et al. (1998) Glucose-induced oxidative stress in vascular contractile cells: comparison of aortic smooth muscle cells and retinal pericytes. Diabetes 47: 801–809
16. Kashiwagi A, Asahina T, Ikebuchi M et al. (1994) Abnormal glutathione metabolism and increased cytotoxicity caused by H2O2 in human umbilical vein endothelial cells cultured in high glucose medium. Diabetologia 37: 264–269
17. Baynes JW (1991) Role of oxidative stress in development of complications in diabetes. Diabetes 40: 405–412
18. Rao GN, Berk BC (1992) Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. Circ Res 70: 593–599
19. Yasunari K, Kohno M, Kano H, Yokokawa K, Minami M, Yoshikawa J (1999) Antioxidants improve impaired insulin-mediated glucose uptake and prevent migration and proliferation of cultured rabbit coronary smooth muscle cells induced by high glucose. Circulation 99: 1370–1378
20. Yasunari K, Kohno M, Kano H, Minami M, Yoshikawa J (2000) Aldose reductase inhibitor improves insulin-mediated glucose uptake and prevents migration of human coronary arter smooth muscle cells induced by high glucose. Hypertension 35: 1092–1098
21. Vinson JA, Staretz ME, Bose P, Kassm HM, Basalyga BS (1989) In vitro and in vivo reduction of erythrocyte sorbitol by ascorbic acid. Diabetes 38: 1036–1041
22. Terashima H, Hama K, Yamamoto R et al. (1984) Effects of a new aldose reductase inhibitor on various tissues in vitro. J Pharmacol Exp Ther 229: 226–230
23. Heasley LE, Johnson GL (1989) Regulation of protein kinase C by nerve growth factor, epidermal growth factor, and phorbol esters in PC12 pheochromocytoma cells. J Biol Chem 264: 8646–8652
24. Yasuda I, Kishimoto A, Tanaka S, Tominaga M, Sakurai A, Nishizuka Y (1990) A synthetic peptide substrate for selective assay of protein kinase C. Biochem Biophys Res Commun 166: 1220–1227
25. Smith PK, Krohn RI, Hermanson GT et al. (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76–85
26. Obrosova IG, Fathallah L, Lang HJ, Greene DA (1999) Evaluation of a sorbitol dehydrogenase inhibitor on diabetic peripheral nerve metabolism: a prevention study. Diabetologia 42: 1187–1194
27. Williamson DH, Lund P, Krebs HA (1967) The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. Biochem J 103: 514–527
28. Masuda T, Dobson GP, Veech RL (1990) The Gibbs-Donnan near-equilibrium system of heart. J Biol Chem 265: 20321–20334
29. Gutmann I, WahlefeLd AW (1974) L-(+)-Lactate determination with lactate dehydrogenase and NAD. In: Berg-

meyer HU (ed) Methods of enzymatic analysis. Vol 3. Academic Press, New York, London, pp 1464–1468
30. Czok R, Lamprecht WB (1974) Pyruvate, phosphoenolpyruvate and D-glycerate-2-phosphate. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Vol 3. Academic Press, New York, London, pp 1446–1451
31. Lou MF, Dickerson JE Jr, Garadi R, York BM Jr (1988) Glutathione depletion in the lens of galactosemic and diabetic rats. Exp Eye Res 46: 517–530
32. Sakakibara F, Hotta N, Koh N, Sakamoto N (1993) Effects of high glucose concentrations and epalrestat on sorbitol and myo-inositol metabolism in cultured rabbit aortic smooth muscle cells. Diabetes 42: 1594–1600
33. Williams B, Howard RL (1994) Glucose-induced changes in Na + /H + antiport activity and gene expression in cultured vascular smooth muscle cells. Role of protein kinase C. J Clin Invest 93: 2623–2631
34. Williams B, Schrier RW (1992) Characterization of glucose-induced in situ protein kinase C activity in cultured vascular smooth muscle cells. Diabetes 41: 1464–1472
35. Inoguchi T, Xia P, Kunisaki M, Higashi S, Feener EP, King GL (1994) Insulin's effect on protein kinase C and diacylglycerol induced by diabetes and glucose in vascular tissues. Am J Physiol 267: E369–E379
36. Kunisaki M, Bursell SE, Umeda F, Nawata H, King GL (1994) Normalization of diacylglycerol-protein kinase C activation by vitamin E in aorta of diabetic rats and cultured rat smooth muscle cells exposed to elevated glucose levels. Diabetes 43: 1372–1377
37. Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W, King GL (1992) Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. Proc Natl Acad Sci USA 89: 11059–11063
38. Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K, King GL (1997) Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. J Clin Invest 100: 115–126
39. Wakasaki H, Koya D, Schoen FJ et al. (1997) Targeted overexpression of protein kinase C ß2 isoform in myocardium causes cardiomyopathy. Proc Natl Acad Sci USA 94: 9320–9325
40. Xia P, Inoguchi T, Kern TS, Engerman RL, Oates PJ, King GL (1994) Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. Diabetes 43: 1122–1129
41. Lee TS, MacGregor LC, Fluharty SJ, King GL (1989) Differential regulation of protein kinase C and (Na,K)-adenosine triphosphatase activities by elevated glucose levels in retinal capillary endothelial cells. J Clin Invest 83: 90–94
42. Yasunari K, Kohno M, Kano H, Yokokawa K, Horio T, Yoshikawa J (1995) Aldose reductase inhibitor prevents hyperproliferation and hypertrophy of cultured rat vascular smooth muscle cells induced by high glucose. Arterioscler Thromb Vasc Biol 15: 2207–2212
43. Griendling KK, Ushio-Fukai M (1998) Redox control of vascular smooth muscle proliferation. J Lab Clin Med 132: 9–15
44. Yasunari K, Kohno M, Kano H, Yokokawa K, Minami M, Yoshikawa J (1997) Mechanisms of action of troglitazone in the prevention of high glucose-induced migration and proliferation of cultured coronary smooth muscle cells. Circ Res 81: 953–962
45. Graier WF, Grubenthal I, Dittrich P, Wascher TC, Kostner GM (1995) Intracellular mechanism of high D-glucose-induced modulation of vascular cell proliferation. Eur J Pharmacol 294: 221–229
46. Harlan JM, Levine JD, Callahan KS, Schwartz BR, Harker LA (1995) Glutathione redox cycle protects cultured endothelial cells against lysis by extracellularly generated hydrogen peroxide. J Clin Invest 73: 706–713
47. Schraufstatter IU, Hinshaw DB, Hyslop PA, Spragg RG, Cochrane CG (1985) Glutathione cycle activity and pyridine nucleotide levels in oxidant-induced injury of cells. J Clin Invest 76: 1131–1139
48. Tesfamariam B (1994) Free Radicals in diabetes endothelial cell dysfunction. Free Radic Biol Med 16: 383–391
49. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T (1995) Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. Science 270: 296–299
50. Curcio F, Pegoraro I, Dello Russo P, Falleti E, Perrella G, Ceriello A (1995) Sod and GSH inhibit the high glucose-induced oxidative damage and the PDGF increased secretion in cultured human endothelial cells. Thromb Haemost 74: 969–973
51. Satoh H, Togo M, Hara M et al. (1997) Advanced glycation endproducts stimulate mitogen-activated protein kinase and proliferation in rabbit vascular smooth muscle cells. Biochem Biophys Res Commun 239: 111–115
52. Lal S, Randall WC, Taylor AH et al. (1997) Fructose-3-phosphate production and polyol pathway metabolism in diabetic rat hearts. Metabolism 46: 1333–1338
53. Hamada Y, Nakamura J, Naruse K, et al. (2000) Epalrestat, an aldose reductase inhibitor, reduces the level of N^{ε}-(carboxymethyl) lysine protein adducts and their precursors in erythrocytes from diabetic patients. Diabetes Care 23: 1539–1544